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Selective and Differential Medium for the Primary Isolation of Members of the *Proteeae*

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A new differential and selective medium for the isolation of members of the *Proteeae*, PIM (*Proteeae* isolation medium) agar, was developed and evaluated. The medium relies on the ability of all members of the *Proteeae* (with the exception of a very few *Morganella morganii* strains) to produce a dark brown pigment in medium containing DL-tryptophan. An additional differential property, tyrosine degradation, was also demonstrated by the medium. Members of the *Proteeae* appeared as dark brown colonies with a halo of clearing of fine tyrosine crystals when cultured on PIM agar. Occasional strains of *Citrobacter* sp. and *Pseudomonas aeruginosa* may degrade tyrosine, but none has the ability to produce dark brown pigmentation on PIM agar. Quantitative recovery studies showed that the addition of 5 mg of clindamycin per liter suppressed gram-positive bacteria without inhibiting any strains of the *Proteeae*. The addition of 100 mg of colistin per liter made the medium highly selective for strains of the *Proteeae*, but approximately 10% of the strains were not isolated, thus making this formulation unsuitable for general surveys of the occurrence of members of the *Proteeae*. PIM agar should aid the investigation of episodes of cross infection caused by members of the *Proteeae* and the isolation of the new species of the *Proteeae* recently described.

Members of the *Proteeae* are well recognized as important nosocomial pathogens (12, 14). They are the most common group of bacteria isolated from cases of nosocomial urinary tract infection associated with long-term urinary catheterization (2, 17). In addition, a number of the *Proteeae* often are resistant to antibiotics commonly used in hospital practice, which may well facilitate their spread within that environment (3, 7, 19). It is evident that there is a need for the continuing study of these bacteria and therefore for a good selective and differential medium for their isolation. Such a medium could be of use in the careful investigation of episodes of cross infection caused by members of the *Proteeae* (particularly if antibiotic markers are unstable in the strain suspected of cross infection). Also, the recent recognition of additional species of the *Proteeae* (5) makes the investigation of their clinical significance of importance; a good primary isolation medium would be useful for those studies.

We describe here a reliable and simple differential and selective medium for the primary isolation of all genera of the *Proteeae* from clinical specimens.

MATERIALS AND METHODS

Bacteria. Eight of the clinically important species-biogroups of the *Proteeae* (*Proteus mirabilis, Proteus vulgaris, Proteus vulgaris* [BG2], *Proteus penneri, Morganella morganii, Providencia rettgeri, Providencia stuartii, Providencia alcalifaciens*) were studied. The total number of strains of the *Proteeae* examined was 189, all of which had been isolated from patients in the United Bristol Hospitals. A further 42 strains of six species of gram-negative bacteria that could possibly be confused with the *Proteeae* were also tested. For details of the species used and their respective number of strains, see Table 2. Members of the *Proteeae* were identified according to the criteria of Farmer and his colleagues (5), and other gram-negative bacteria were identified by using the API 20E and API 20NE (equivalent to the API Rapid NFT; Analytab Products, Montalieu-Varcieu, France).

PIM medium. *Proteeae* isolation medium (PIM) consisted of L-tyrosine (4 g) and DL-tryptophan (5 g) added to 1,000 ml of Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing extra agar (8 g) (to inhibit swarming) which was autoclaved for 15 min at 121°C. The medium was allowed to cool to 53°C, and 5 mg of clindamycin sulfate (The UpJohn Co., Kalamazoo, Mich.) was added and mixed to ensure even distribution of the tyrosine crystals before the medium was poured into petri dishes. It was found that better results were obtained if plates were poured to give a thin layer of medium. The medium was stored at 4°C for 24 h before use to allow complete precipitation of the tyrosine. The medium remained stable for up to 2 weeks when stored at 4°C.

On PIM medium, after 48 h of incubation at 37°C, members of the *Proteeae* appear as dark brown colonies surrounded by a zone of clearing in the medium. Other bacterial colonies are colorless with no zone of clearing, although with bacteria that are able to degrade tyrosine (*Pseudomonas aeruginosa* and some strains of *Citrobacter freundii* and *Citrobacter diversus*), a zone of clearing appears around a colorless colony.

Concentrations of antibiotics in PIM and inhibitory effect of PIM agar on the *Proteeae*. It was decided to prepare PIM agar in two forms, a relatively nonselective medium (containing clindamycin only) for the isolation of colistin-susceptible and -resistant strains of the *Proteeae* and a highly selective medium containing clindamycin and colistin for the isolation of colistin-resistant strains of the *Proteeae*. One strain each of the clinically significant *Proteeae* and a number of other bacterial species likely to be found in clinical specimens (as shown in Table 1) were examined by preparing 10 10-fold dilutions of 18-h infusion broth cultures (BBL) in sterile saline.

Each of the dilutions was then counted by a standard

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TABLE 1. Effect of incorporation of clindamycin and colistin alone and together in PIM on viable counts of the *Proteeae* and other enteric bacteria

Organism	No. of bacteria (CFU/ml)[a] grown on:			
	CLED[b]	PIM + clindamycin[c]	PIM + colistin[d]	PIM + colistin and clindamycin
Proteus mirabilis	3.5×10^8	4.0×10^8	6.0×10^7	7.0×10^7
Proteus vulgaris	8.0×10^8	7.5×10^8	1.0×10^9	5.0×10^8
Providencia stuartii	1.5×10^8	5.0×10^7	7.0×10^7	3.0×10^7
Providencia rettgeri	1.0×10^9	9.0×10^8	1.0×10^9	9.0×10^8
Morganella morganii	1.0×10^9	8.0×10^8	8.5×10^8	8.5×10^8
Staphylococcus epidermidis	1.0×10^8	NG[e]	NG	NG
Escherichia coli NCTC 10418	8.5×10^8	NG	NG	NG
Streptococcus faecalis	8.5×10^8	NG	6.0×10^8	NG
Klebsiella aerogenes	1.0×10^9	9.0×10^8	NG	NG

[a] Viable counts are the means of four determinations for that species.
[b] Cystine-lactose-electrolyte-deficient agar (Oxoid).
[c] Antibiotic concentration was 5 mg of clindamycin per liter.
[d] Antibiotic concentration was 100 mg of colistin per liter.
[e] NG, No growth.

method (13) on duplicate plates of PIM containing 5, 25, or 50 mg of clindamycin per liter and of PIM with 5, 25, or 50 mg of clindamycin per liter and 20, 100, or 200 mg of colistin sulfate (sterile powder supplied by Pharmax Ltd., Bexley, England) per liter. All the dilutions were also repeated on cystine-lactose-electrolyte-deficient agar (Oxoid Ltd., Basingstoke, England) to obtain a control viable count.

Susceptibility to colistin. Susceptibility to colistin of each of the *Proteeae* isolates was tested by a standard controlled disk technique (18) with 100-μg disks (Mast Laboratories Ltd., Liverpool, England).

Recovery of isolates of the *Proteeae* and other bacteria from PIM. Isolates of the *Proteeae* and other bacteria were plated onto PIM agar containing clindamycin to produce isolated colonies, and the plates were incubated at 37°C for 48 h. Colonies were assessed for production of dark brown pigmentation and degradation of tyrosine (zone of clearing, 2 to 4 mm in diameter). It was found that the concentration of agar used in PIM was adequate to prevent swarming of *Proteus* sp. in all cases, provided the plates were properly dried before use.

RESULTS

A comparison of various combinations of clindamycin and colistin showed that 5 mg of clindamyin per liter and 100 mg of colistin per liter provided maximum inhibition of gram-positive and gram-negative bacteria, whereas no inhibition of any colistin-resistant members of the *Proteeae* was noted. Strains found to be susceptible by disk testing failed to grow on PIM containing 20 mg of colistin per liter. The use of colistin would therefore result in the failure to recover 9.0% of the *Proteeae* strains, and our suggested formulation of PIM does not include colistin. If isolation of colistin-resistant strains of the *Proteeae* is desired, concentrations of clindamycin greater than 5 mg/liter should not be used, because concentrations of 25 and 50 mg of clindamycin per liter together with 100 mg of colistin per liter failed to recover some strains of the *Proteeae* (data not shown). The use of 20 mg of colistin per liter was not found to be ideal because colistin-susceptible strains of the *Proteeae* failed to grow, but some strains of the family *Enterobacteriaceae* did grow. Increasing the colistin concentration to 100 mg/liter eliminated these strains, while all colistin-resistant strains of the *Proteeae* grew (data not shown). Mean viable counts from four determinations are given in Table 1. To improve clarity, only values for the finally selected antibiotic concentrations are shown.

Susceptibility to colistin. The susceptibility to colistin of each of the species of the *Proteeae* tested is shown in Table 2. The MIC of colistin for the control strain of *Escherichia coli* (NCTC 10418) used was 0.5 mg/liter, and susceptible members of the *Proteeae* produced almost concentric zones of inhibition, implying a MIC of colistin of approximately 2 to 4 mg/liter.

The overall susceptibility of the 189 isolates of the *Proteeae* examined was 9.0%.

Recovery of strains of the *Proteeae* and other bacteria from PIM agar. The results of the direct plating of strains of the *Proteeae* and other gram-negative bacteria on PIM containing 5 mg of clindamycin per liter are shown (Table 2). All of the strains of the *Proteeae* examined, with the exception of one strain of *M. morganii*, produced a dark brown pigmentation of the colony. None of the other bacteria produced this pigment. Of the other bacteria tested, only *Pseudomonas aeruginosa* degraded tyrosine (80% of the isolates); all members of the *Proteeae* with the exception of 53% of the *M. morganii* isolates degraded tyrosine.

DISCUSSION

Very few examples of selective media for the primary isolation of members of the *Proteeae* have been reported in the literature. A selective medium relying on lithium chloride, sodium thiosulfate, and sodium citrate has been described for the primary isolation of *Proteus* spp. (20). However, 6% of *P. mirabilis* and 8% of *M. morganii* failed to grow on the medium despite an inoculum of approximately 10^3 CFU; also, no mention was made of the suitability of the medium for the isolation of *Providencia* spp. A complex medium including a number of dyes, alanine, and potassium nitrate incorporated into MacConkey agar has been reported for the differentiation of *Salmonella* and *Shigella* species from *Proteus* spp. (10). Differentiation from other non-lactose-fermenting bacteria relied on distinguishing between the bluish grey colonies of *Proteus* spp. and the white to grey colonies of the *Enterobacteriaceae*; again, no data were provided on suitability for isolating *Providencia* spp. Primary isolation media incorporating the antibiotic colistin (or polymyxin B) have also been reported (4, 8, 14). Use of these media is permissible if a particular strain known to be colistin resistant is to be isolated. However, if the colistin susceptibility of clinical isolates is routinely tested, quite large numbers of colistin-susceptible strains (particularly *P. vulgaris*) may be found (6). In another study of colistin susceptibility in known species of the *Proteeae*, between 11 and 60% were found to be susceptible to colistin (11). To miss this proportion of isolates in a thorough survey would be unacceptable; therefore, a medium which is selective without the use of colistin would be useful.

Confirmation of the occurrence of colistin-susceptible *Proteeae* among clinical isolates is provided by the figures in

TABLE 2. Numbers of the *Proteeae* and other enteric bacteria producing dark brown pigment and degrading tyrosine when inoculated on PIM after incubation for 48 h at 37°C

Organism	No. of strains	Differential property of bacteria grown on PIM[a]		% of strains with both differential properties	No. of strains of the *Proteeae* susceptible to colistin
		Production of pigment	Degradation of tyrosine		
Proteus mirabilis	47	47 (100)	47 (100)	100	1
Proteus vulgaris	21	21 (100)	21 (100)	100	4
Proteus penneri	16	16 (100)	16 (100)	100	4
Proteus vulgaris BG2	10	10 (100)	10 (100)	100	1
Morganella morganii	17	16 (94)	8 (47)	94	2
Providencia stuartii	39	39 (100)	39 (100)	100	4
Providencia rettgeri	21	21 (100)	21 (100)	100	1
Providencia alcalifaciens	18	18 (100)	18 (100)	100	0
Pseudomonas aeruginosa	25	0	20 (80)	0	
Pseudomonas fluorescens	1	0	0	0	
Pseudomonas maltophilia	3	0	0	0	
Citrobacter freundii	8	0	0	0	
Acinetobacter sp.	3	0	0	0	
Alcaligenes sp.	2	0	0	0	

[a] Numbers in parentheses are percentages of isolates positive.

Table 2, with 9.0% of strains isolated being susceptible to colistin. Colistin-resistant strains of the *Proteeae* (as defined by controlled disk testing) were found to grow adequately on media containing 100 mg of that antibiotic per liter. The effect of colistin on the viable counts of five colistin-resistant examples of the commonly encountered *Proteeae* plated on media containing 100 mg/liter is shown in Table 1. There is no detectable reduction in viable counts. It was our experience that colistin-susceptible strains of the *Proteeae* failed to grow at all on colistin-containing media, even on media containing 20 mg of colistin per liter (see Results). Not incorporating colistin in a selective medium for the recovery of members of the *Proteeae* means that the *Enterobacteriaceae* will grow on the plate and might obscure small numbers of the *Proteeae*. In practice we did not find this to be a great problem because the brown coloration of colonies of the *Proteeae* enabled them to be subcultured to yield isolated colonies. The inclusion of clindamycin eliminated gram-positive bacteria. The effects of the various antibiotic combinations on single isolates of *Staphylococcus epidermidis*, *Streptococcus faecalis*, and *Klebsiella aerogenes* and on the control *E. coli* NCTC 10418 are shown in Table 1. Interestingly, the very sensitive *E. coli* tested was eliminated by the clindamycin, and the *K. aerogenes* was unaffected. We found the addition of clindamycin very helpful in eliminating the gram-positive flora, thus facilitating the detection of small numbers of the *Proteeae*.

The production of a deep brown pigment by the *Proteeae* on media containing tryptophan was reported in 1964 when tryptophan was suggested for the identification of strains of the *Proteeae* rather than as a primary isolation medium (15). The authors examined 2,095 isolates of 41 species of the *Enterobacteriaceae* and found the pigment to be exclusively produced by the *Proteeae*. As in this study, three isolates of *M. morganii* failed to give a positive result, and pigment production was reported as generally less intense in this species. We combined this useful property of the *Proteeae* with another phenomenon, tyrosine degradation, which is reported to occur only in the *Proteeae* and in some strains of *Citrobacter* spp. (16). It has subsequently been reported that tyrosine degradation is also useful for differentiating *Peptostreptococcus anaerobius* (1). We found that 80% of our strains of *Pseudomonas aeruginosa* have the ability to degrade tyrosine, although the limited number of other species of *Pseudomonas* examined did not. We found that the *Proteeae* produced obvious and moderate-sized zones of clearing in PIM. That fact, combined with the dark brown pigmentation which all the *Proteeae* species produce (except rare *Morganella* strains), made single colonies of the *Proteeae* very easy to distinguish from other bacteria. PIM has been used effectively by us in an epidemiological study of postprostatectomy urinary infection in catheterized patients, during which small numbers of the *Proteeae* in mixed cultures were readily distinguished (A. McCormick, B.Sc. thesis, University of Bristol, Bristol, England). When PIM is combined with suitable liquid enrichment media, which have been reported to greatly enhance the recovery of strains of the *Proteeae* (9, 14), the recovery of small numbers of various species of the *Proteeae* (including the recently defined species) from specimens such as feces should be greatly improved.

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